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TECHNICAL MANUSCRIPT 375

# EFFECT OF EDTA AND PHOSPHOLIPASES ON PERMEABILITY OF ESCHERICHIA COLI AS MEASURED BY β-GALACTOSIDASE ACTIVITY

Milton W. Slein
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JULY 1967

DEPARTMENT OF THE ARMY
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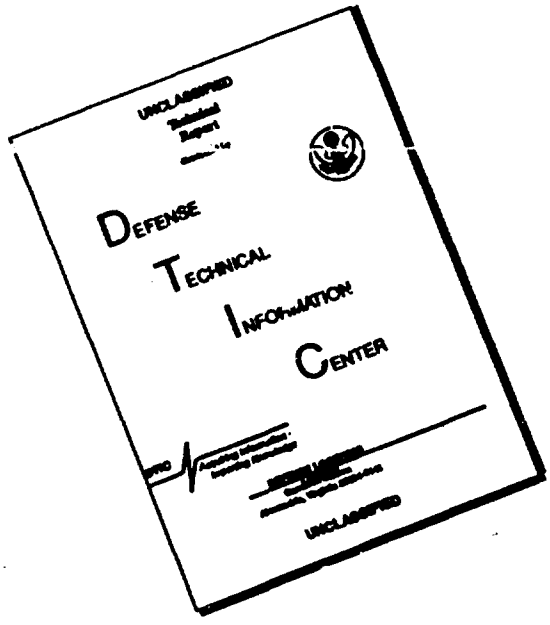


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TECHNICAL MANUSCRIPT 375

# EFFECT OF EDTA AND PHOSPHOLIPASES ON PERMEABILITY OF *ESCHERICHIA COLI* AS MEASURED BY β-GALACTOSIDASE ACTIVITY

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BIOLOGICAL SCIENCES LABORATORY

Project 1C014501B71A

July 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

## ABSTRACT

A permeaseless mutant of Escherichia coli that produces β-galactosidase constitutively was treated briefly with ethylenediaminetetraacetate (EDTA) and then with the phospholipases of Bacillus cereus. An increase in cell permeability and lysis was indicated by an increase in β-galactosidase activity and a decrease in absorbancy of the cell suspension. The susceptibility of the cells to attack by EDTA and the phospholipases was markedly affected by the age of the cells when harvested. The results suggest that the permeability changes are associated with the activity of a phospholipase that specifically degrades phosphatidyl ethanolamine. A sonic-treatment method, which is independent of the age of E. coli cells when harvested, is described for determining their total β-galactosidase content.

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# I. INTRODUCTION

This preliminary study of the effect of the phospholipases of Bacillus cereus[1,2] on bacterial cell membranes has as an ultimate goal the obtaining of information about the role of phospholipids in cell membrane structure and function. We used a cryptic mutant strain of Escherichia coli that produces β-galactosidase constitutively but lacks the permease necessary for transport of galactoside substrates for the enzyme. Any increase in permeability of the membrane to the β-galactosidase or its substrates is reflected by increased enzyme activity. Some of the properties of a sensitive test system are reported, along with results suggesting that the phospholipases increase permeability by degrading phosphatidyl ethanolamine (PTE), the principal phospholipid constituent of the membranes of E. coli.[3]

# II. MATERIALS AND METHODS

## A. PREPARATION OF CELL SUSPENSIONS

A culture of E. coli ML35 ($i^- z^+ y^-$)* was grown at 37 C in shaken flasks containing the minimal glycerol medium of Brock and Brock.[4] An inoculum was prepared by incubating a loopful of cells from a slant (0.8% Difco nutrient broth, 0.3% Difco yeast extract, and 2% Difco agar) in 25 ml of medium in a 250-ml Erlenmeyer flask for 24 hours. About 2 to 3 ml of this culture were used to inoculate 100 ml of medium in a 1-liter Erlenmeyer flask so that the initial absorbancy at 420 mμ was 0.5 measured on a Beckman DB spectrophotometer. Cells were harvested at intervals, washed twice with cold distilled water, and suspended in water so that a small sample had an absorbancy of 0.50 to 0.55 after being diluted 1:100. Cells harvested at 3 to 4 hours clumped while being suspended. Any small clumps were removed by filtering through a thin layer of glass wool. The cell suspensions were stored at 5 C.

## B. TREATMENT OF CELLS AND MEASUREMENT OF β-GALACTOSIDASE

Immediately before use, a portion of the cell suspension was diluted with an equal volume of cold 0.1 M tris(hydroxymethyl)aminomethane (pH 7.5), and 0.2-ml samples were treated with 0.1 ml 0.004 M ethylenediaminetetraacetate (EDTA) for exactly 3 minutes at 37 C. The EDTA reaction was interrupted by adding 0.05 ml of 0.02 M $MnCl_2$. One minute later, 0.05 ml

* Supplied by H.V. Rickenberg.

of phospholipase or other test solution was added. Incubation at 37 C was continued for 10 to 20 minutes. We then added 3.6 ml of a cold solution (PMSH) of 0.01 M phosphate (pH 7.2), 0.9% NaCl, 0.0001 M $MnCl_2$, and 0.05 M 2-mercaptoethanol. A similar buffer (PM2) was described by Horiuchi et al.[5] for use in the measurement of β-galactosidase activity. One-tenth ml of the treated cells was further diluted with 4.9 ml of cold PMSH; duplicate 1-ml samples were used for β-galactosidase determination. The original washed cell suspension had been diluted 1:2000 at this point. The samples were warmed for exactly 2 minutes in a water bath at 37 C before addition of 0.1 ml of a solution of 4 mg o-nitrophenyl-β-D-galactoside (ONPG)* in 1 ml 0.25 M phosphate buffer, pH 7.2. After 5 to 10 minutes, the reaction was stopped by adding 0.5 ml 1 M $Na_2CO_3$; the samples were then read in a Beckman DB spectrophotometer at 420 mμ with water as the reference solution. Mercaptoethanol was not present during treatment of the cells with EDTA and the phospholipases because it inhibited the effect of the latter.

Total β-galactosidase activity was determined after sonic treatment of the diluted cells (1:2000 in PMSH). Ten ml of cells were placed in a 25- x 75-mm celluloid tube in an ice bath and treated with the flat-tipped 0.5-inch-diameter step horn of a Model LS-75 Branson Sonifier with maximal output at 6 amp. The tip was immersed as far as possible into the cell suspension. The cells were shaken for 1 minute in the ice between each of four 30-second treatments to keep the temperature below 30 C. Under these conditions practically maximal β-galactosidase activity was released with cells harvested at all stages of growth. Using a smaller volume of cell suspension or immersing the probe less deeply decreased the efficiency of cell disruption because a vigorous "rolling" action occurred.

Cellulose triacetate membrane filters were used to determine extracellular β-galactosidase because they did not adsorb any significant enzyme; filters containing mixed cellulose esters (Millipore, type HA) removed most of the enzyme from dilute solutions. Metricel GA-6 filters, 13 mm in diameter with 0.45-μ pores, were obtained from the Gelman Instrument Co., Ann Arbor, Mich. Millipore Celotate EH filters might also be used, but they were not tested. Cell samples were taken into a syringe with a stainless steel cannula and were filtered by ejecting the samples through filters in Swinny hypodermic adapters.**

* Obtained from Sigma Chemical Co., St. Louis, Mo.
** Millipore Filter Corp., Watertown, Mass.

### C. PHOSPHOLIPASES AND ANTISERUM

The crude phospholipases of *B. cereus* and partially purified fractions were prepared as described previously.[1,2] *Clostridium perfringens* α-toxin (phospholipase C) was purchased from CalBiochem, Los Angeles, Calif. Phospholipase activity was determined as previously described, but the phospholipids were emulsified directly in 0.2 M phosphate (pH 7) so that the buffer concentration of the emulsion was 0.2 M instead of 0.133 M.[2] Most of the phospholipase activity of crude preparations from *B. cereus* was destroyed by autoclaving in sealed ampoules for 15 minutes at 121 C.

Antiserum to the phospholipases of *B. cereus* was prepared by serial subcutaneous injection of an emulsion of the crude phospholipases in Freund's complete adjuvant (Difco) into rabbits. The gamma globulin fractions of normal serum and antiserum were precipitated with 0.33 saturated $(NH_4)_2SO_4$[6] and dialyzed against cold 0.9% NaCl. Crude phospholipase was incubated with the globulins or with NaCl for 30 minutes at 37 C and then overnight at 5 C. Precipitates were removed by centrifugation, and the clear supernatant solutions were tested for their effect on *E. coli* cells and for residual phospholipase activity.

### D. OTHER PROCEDURES

Protein was determined by the method of Lowry et al.[7]

After brief sonic treatment of *E. coli* cells at 0 C, phospholipids were extracted by the method of Bligh and Dyer[8] as described for erythrocytes.[2] Phospholipids were determined as previously reported.[2]

## III. RESULTS

### A. PRETREATMENT WITH EDTA

The ability of phospholipases to increase the permeability of *E. coli* cells depended upon preliminary treatment of the cells with EDTA; galactosidase activity increased only slightly in its absence. The use of both EDTA and lysozyme for preparing the cells resulted in too much lysis that was difficult to control. The effect of time of treatment with EDTA before treatment for 10 minutes with crude phospholipases is shown in Figure 1 for cells harvested after 4.5 hours of growth. Treatment of 0.75 ml of buffered cells with 0.15 ml water or EDTA (final concentration 0.00133 M) was begun at 0 C, 30 seconds before taking initial 0.1-ml samples into tubes containing 0.02 ml 0.02 M $MnCl_2$ at 37 C. The rest of the mixtures were incubated at 37 C, and subsequent

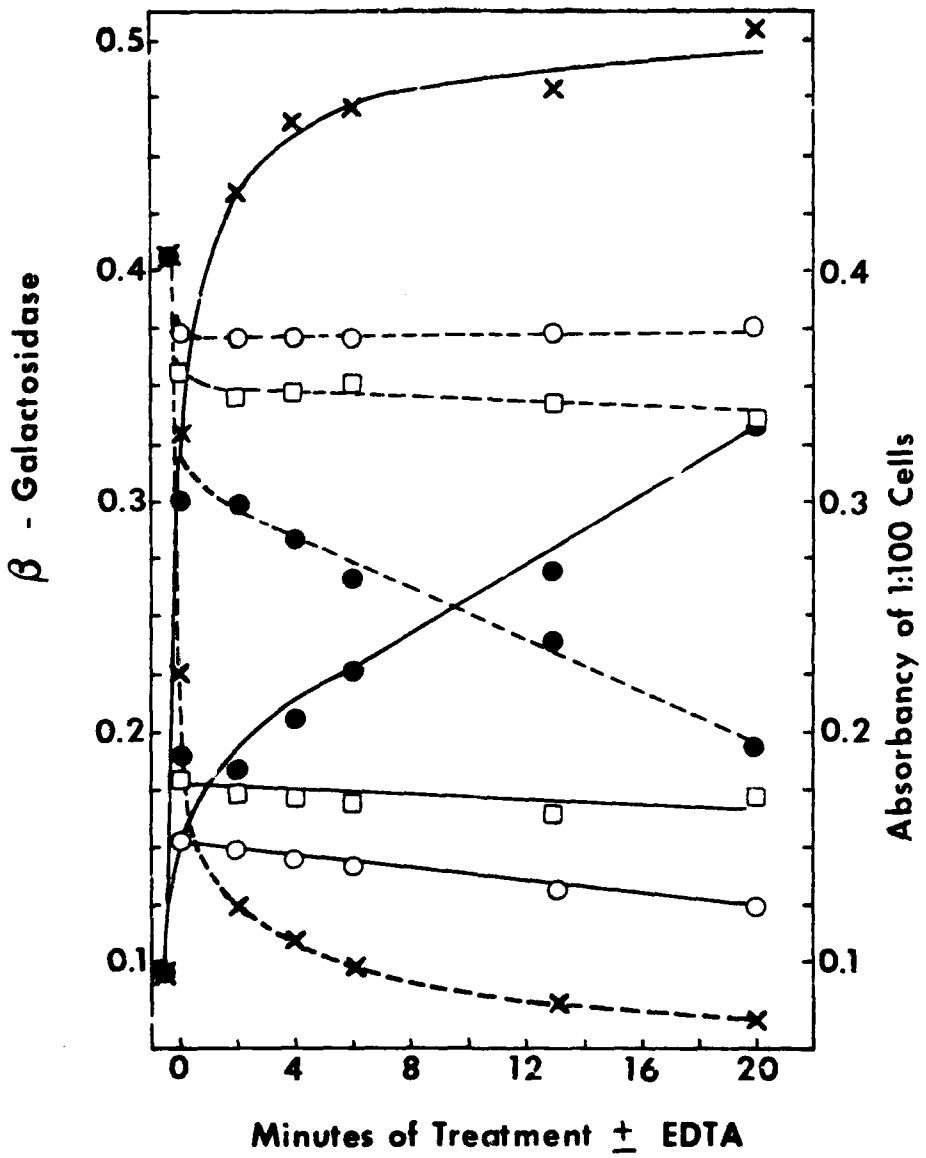


Figure 1. Effect of Time of Treatment of E. coli With or Without EDTA Prior to Treatment for 10 Minutes With or Without Phospholipases. Cells were harvested at 4.5 hours. Treatment began at 0 C at minus 0.5 minute and samples were placed in a 37 C bath at 0 minute (see text for details). Solid lines: galactosidase activity; dashed lines: absorbancy of 1:100 dilution of cells. ●: cells treated with EDTA only; X: cells treated with EDTA and phospholipases; □: cells incubated without EDTA, then treated with phospholipases; O: cells incubated without EDTA or phospholipases.

0.1-ml samples were taken into tubes of warmed $MnCl_2$ at intervals. One minute later, 0.02 ml water or crude phospholipases (16 µg protein) was added to the Mn-treated samples, which were then incubated for 10 minutes before diluting with cold PMSH. Duplicate 1-ml samples of the 1:2000 cells were used for galactosidase determination. Treatment with EDTA alone resulted in an increase of galactosidase activity and a decrease in the absorbancy of the cells. This was markedly affected by subsequent treatment with the phospholipases; the best differential effect occurred after approximately 2 to 4 minutes with EDTA. The addition of $Mn^{++}$ was essential for a good effect; otherwise the EDTA continued to act and also inhibited phospholipase activity. Cells incubated without EDTA had relatively stable galactosidase activity that was only slightly increased by subsequent treatment with the crude phospholipases for 10 minutes. Control cells that were kept in an ice bath until diluted for galactosidase determination gave a value of 0.097. A 1:100 dilution of the untreated control cells had an absorbancy of 0.407.

## B. EFFECT OF TIME OF TREATMENT AND CELL AGE

The effect of various times of treatment of 4.5-hour cells with the crude phospholipases after exposure to EDTA for 3 minutes is shown in Figure 2. The data also show the stability of galactosidase during incubation at 37 C for 20 minutes after interrupting the EDTA action with $Mn^{++}$. The increased galactosidase activity caused by the phospholipases was not merely due to cell lysis but also to an increase in permeability to the substrate ONPG. This is evident in Table 1 with cells harvested after growth for 4, 8, and 24 hours. Treatment with EDTA for 3 minutes and then with the undenatured crude phospholipases for 20 minutes resulted in a 7-fold stimulation of the over-all galactosidase activity of 4-hour cells, 3.3-fold of 8-hour cells, and only 1.2-fold of 24-hour cells. The 4-hour and 8-hour cells also showed a marked increase in intracellular galactosidase activity. A larger increase of extracellular enzyme was obtained with the 4-hour cells than with the 8-hour cells because of a more extensive lysis of the younger cells by the treatment. This is indicated by the decreases in absorbancies of 1:40 dilutions of the treated cells. The effect of heat-inactivated phospholipases was insignificant. After storage for 1 week at 5 C, the cells gave a significantly lower differential response to the phospholipases, apparently because of an increase in permeability.

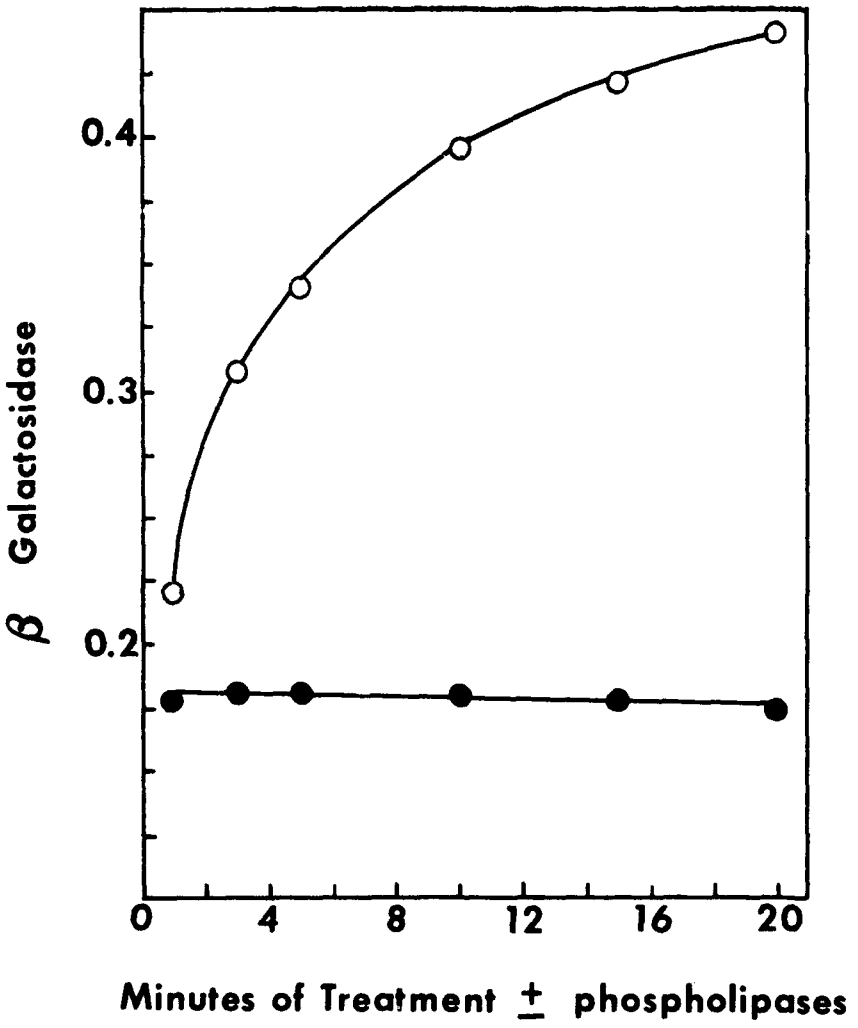


Figure 2. Effect of Time of Treatment of E. coli With or Without Crude Phospholipases of B. cereus after Treatment with EDTA for 3 Minutes. Cells were harvested at 4.5 hours. ●: cells treated with EDTA only; ○: cells treated with EDTA and phospholipases (40 μg protein).

TABLE 1. DISTRIBUTION OF INTRA- AND EXTRACELLULAR GALACTOSIDASE AFTER PHOSPHOLIPASE TREATMENT OF E. COLI CELLS HARVESTED AT DIFFERENT TIMES

| Time Harvested, hr | Treatment[a] | Galactosidase Activity[b] Intracellular, absorbancy | Galactosidase Activity[b] Extracellular, absorbancy | 1:40 Cells after Treatment, absorbancy |
|---|---|---|---|---|
| 4 | EDTA alone | 0.023 | 0.035 | 1.100 |
| | EDTA + autoclaved phospholipases | 0.018 | 0.036 | 1.100 |
| | EDTA + native phospholipases | 0.139 | 0.269 | 0.540 |
| 8 | EDTA alone | 0.014 | 0.016 | 1.100 |
| | EDTA + autoclaved phospholipases | 0.020 | 0.015 | 1.100 |
| | EDTA + native phospholipases | 0.072 | 0.026 | 1.025 |
| 24 | EDTA alone | 0.015 | 0.014 | 1.150 |
| | EDTA + autoclaved phospholipases | 0.016 | 0.014 | 1.125 |
| | EDTA + native phospholipases | 0.019 | 0.016 | 1.125 |

a. Treatment with EDTA for 3 minutes at 37 C, then for 20 minutes without or with crude phospholipases (40 μg protein).

b. This does not represent the total galactosidase content of the cells, but the over-all activity measurable after treatment. The intracellular portion was calculated by subtracting the extracellular value obtained after removing the cells by filtration.

The marked differences in susceptibility to phospholipase obtained with cells harvested at various times are shown more dramatically in Figure 3. Under the culture conditions used, the stationary phase was reached in about 7 hours. All control cells incubated at 37 C without EDTA or phospholipase treatment had low galactosidase activity of 0.060 to 0.073. Maximal galactosidase activity after treatment with EDTA plus crude phospholipase was obtained with cells harvested at 3.5 hours. Susceptibility to stimulation by brief treatment with EDTA alone was also more evident with the younger cells. The total galactosidase content of the cells analyzed in Figure 3 tended to increase somewhat during growth (Table 2). Therefore, the results in Figure 3 reflect changes in the susceptibility of the cells to EDTA and the phospholipases, not merely differences in galactosidase content.

## C. EFFECT OF PHOSPHOLIPASE CONCENTRATION

The effect of phospholipase concentration on EDTA-treated 3.5-hour cells is given in Figure 4. As few as 2.5 μg of crude phospholipase protein almost doubled the galactosidase activity and 40 μg stimulated the activity more than fourfold. Lysis amounted to about 60%, as indicated by the decrease in absorbancy of a 1:40 dilution of the cells after treatment with 40 μg of phospholipase. The crude phospholipase itself had no galactosidase activity.

## D. MEASUREMENT OF TOTAL β-GALACTOSIDASE CONTENT OF CELLS

The release of the total galactosidase from cells of different culture ages by sonic treatment is illustrated in Figure 5. Eighty-seven per cent of maximal activity of 4-hour cells (log phase) was detected after sonic treatment for 15 seconds, but only 51% was obtained with 8-hour cells (stationary phase). Maximal activity of 8-hour cells was obtained after four treatments of 30 seconds each. With 4-hour cells, maximal activity occurred after two 30-second treatments and decreased only 3% after four more treatments. For this reason, total galactosidase activity has been estimated after four 30-second treatments. Other tests demonstrated that approximately 95% of the galactosidase was extracellular after two 30-second treatments even with cells harvested after growth for 24 hours. Little or no loss of cell-free galactosidase occurred under these conditions of sonic treatment. The total galactosidase content of approximately equal amounts of cells harvested after different times of growth is compared in Table 2. The cell concentrations before sonic treatment are indicated by the absorbancies of 1:100 dilutions. Duplicate samples gave excellent results, and the total galactosidase content of cells increased about 30% during growth from 3 to 8 hours. The decreasing galactosidase activities of the untreated cells may indicate that crypticity increases during growth.

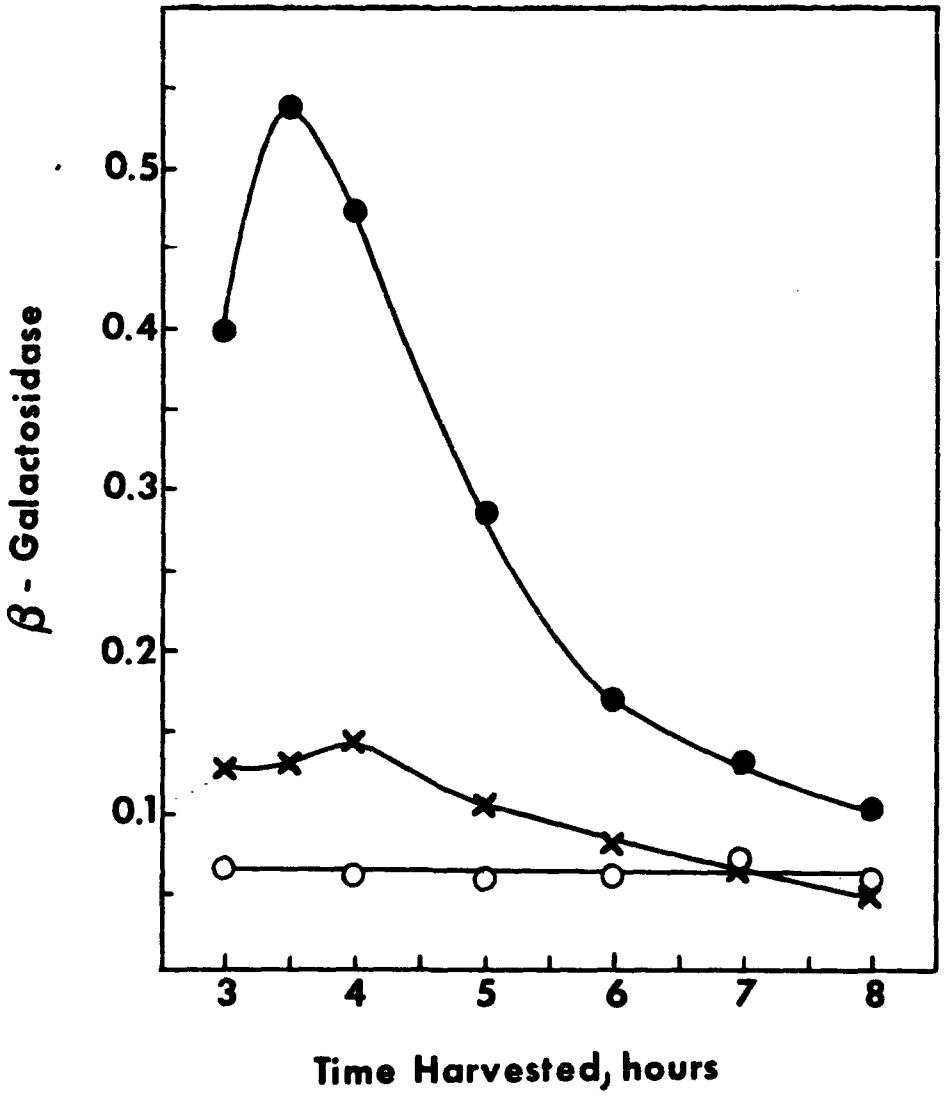


Figure 3. Effect of Culture Age of E. coli Cells on their Susceptibility to Treatment with EDTA Alone or to EDTA Followed by Phospholipases. O: cells incubated without EDTA or phospholipases; X: cells treated for 3 minutes with EDTA and 20 minutes without phospholipases; ●: cells treated for 3 minutes with EDTA and 20 minutes with the crude phospholipases of B. cereus (40 μg protein).

TABLE 2. TOTAL GALACTOSIDASE CONTENT OF E. COLI ML35 DURING GROWTH

| Time Harvested,[a] hr | Concentration of 1:100 Un-Treated Cells, absorbancy | Galactosidase Activity of 1:2000 Cells | |
|---|---|---|---|
| | | Untreated, absorbancy | Sonic-Treated,[b] absorbancy |
| 3 | 0.492 | 0.099 | 0.668 |
| 4 | 0.485 | 0.092 | 0.719 |
| 5 | 0.473 | 0.084 | 0.794 |
| 6 | 0.462 | 0.061 | 0.855 |
| 7 | 0.478 | 0.067 | 0.790 |
| 8 | 0.488 | 0.054 | 0.860 |

a. The same samples as those used for Figure 3.
b. Treated four times for 30 seconds each. Values are averages of duplicate samples.

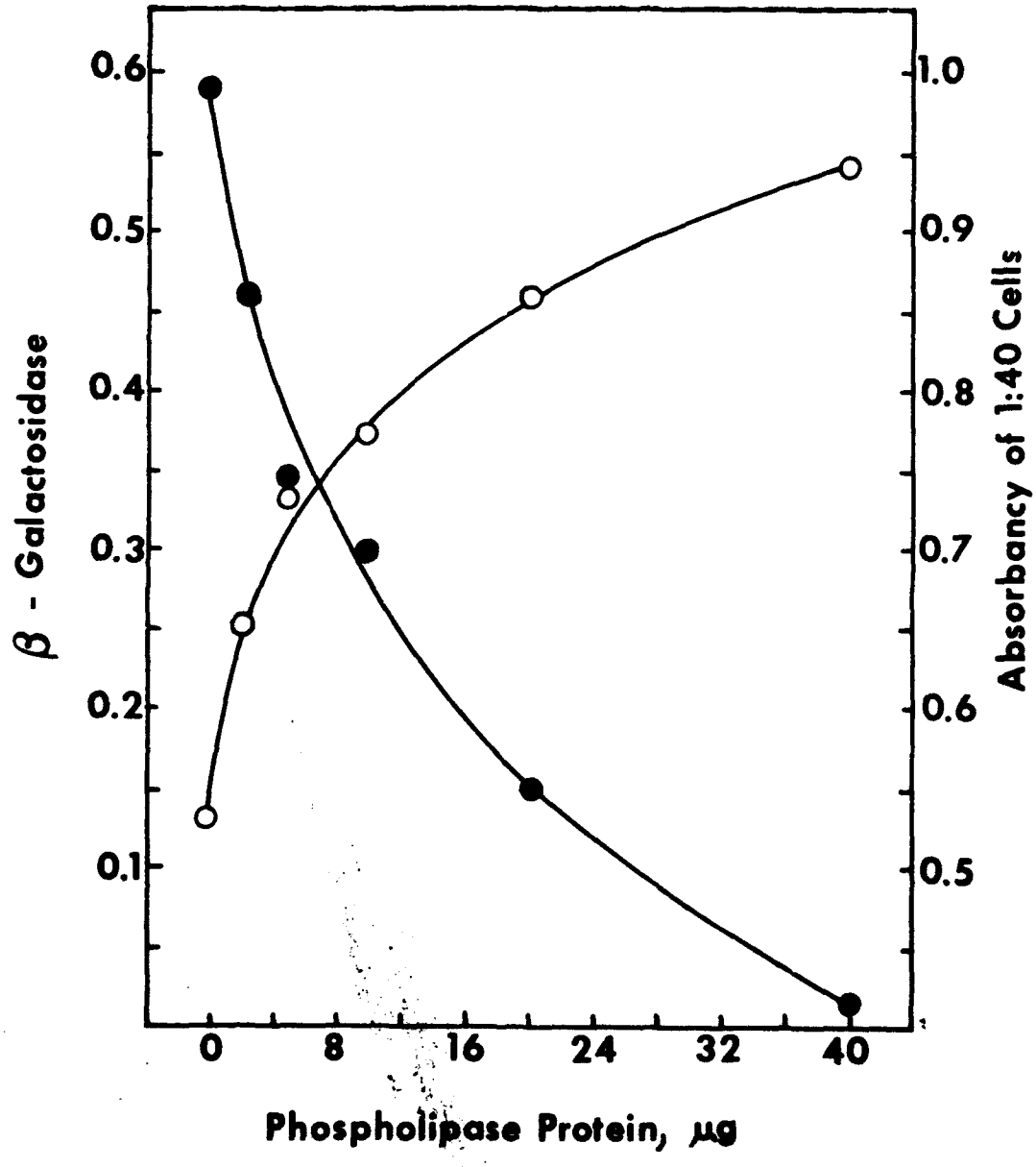


Figure 4. Effect of Various Amounts of the Crude Phospholipases of B. cereus on E. coli Cells. The E. coli cells were harvested at 3.5 hours and treated for 3 minutes with EDTA and for 20 minutes with the phospholipases. O: β-galactosidase activity; ●: absorbancy of 1:40 dilution of cells.

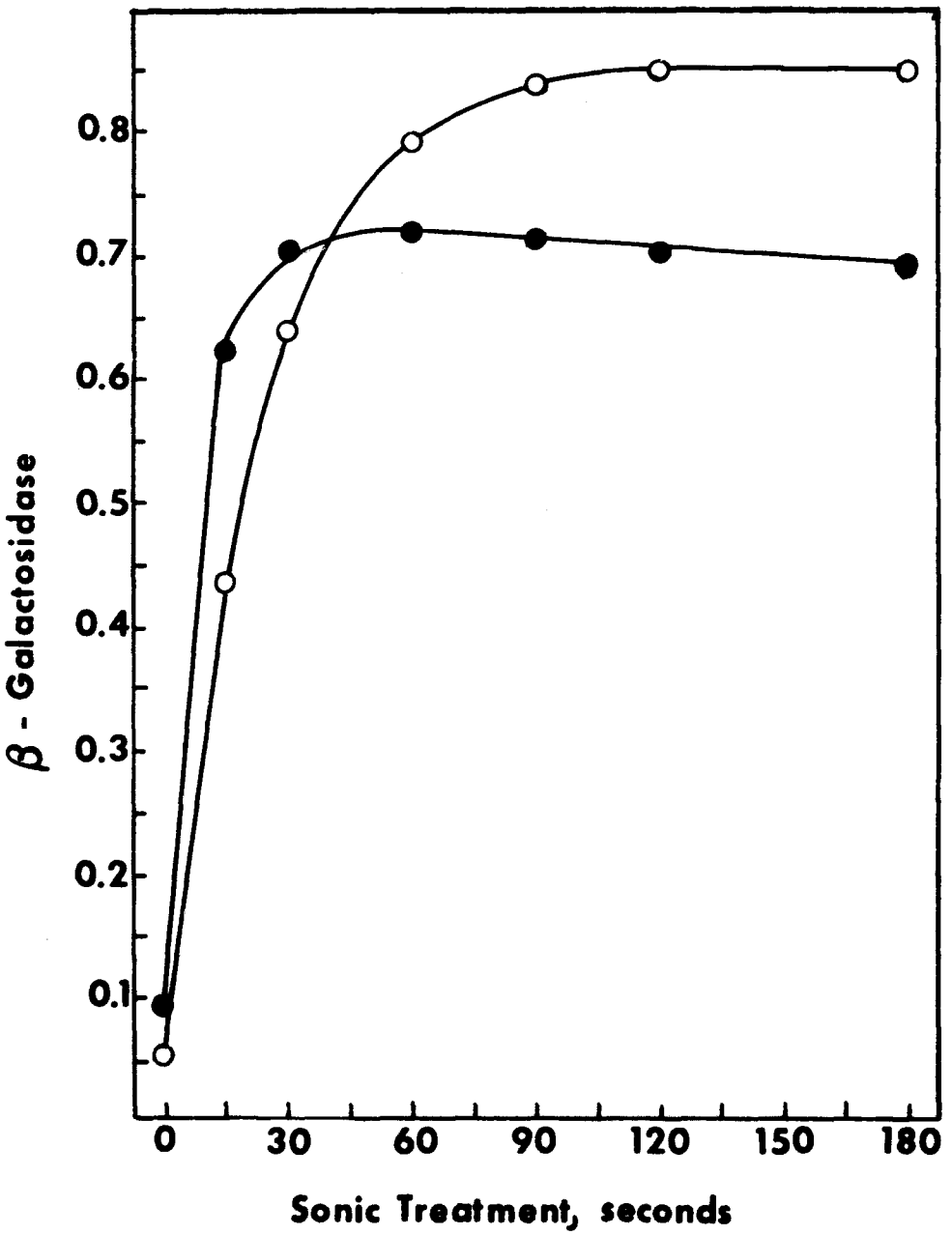


Figure 5. Effectiveness of Sonic Treatment for the Determination of Total β-galactosidase in E. coli Cells Harvested at Different Times. Sonic treatments at 0 C for 15- or 30-second intervals with time allowed for cooling between treatments. ●: 4-hour cells; ○: 8-hour cells.

During storage of the washed 4- and 8-hour cells for 3 weeks at 5 C, the total galactosidase content decreased about 23% and 16%, respectively. Incubation of these aged cells for 1 hour at 37 C in the presence or absence of PMSH before sonic treatment resulted in no significant effect on the galactosidase content of the 8-hour cells. However, the 4-hour cells showed a loss of about 43% during incubation in the absence of PMSH and a 15% increase in the presence of PMSH. The latter effect may have been produced by an activation of galactosidase by the mercaptoethanol in the presence of $Mn^{++}$.[9] The concentrations of thiol and $Mn^{++}$ were the same in all samples during galactosidase measurement. In tests with crude, cell-free galactosidase, enzyme activity did not change significantly during incubation for 1 hour at 37 C in the presence of PMSH.

## E. SPECIFICITY OF THE PHOSPHOLIPASE EFFECT

Antiserum to the crude phospholipases inhibited the action of the phospholipases on E. coli cells as well as their activities, as measured with an emulsion of soybean phospholipids to which sphingomyelin (SPH) had been added (Table 3). Normal serum gamma globulin inhibited the effect of the phospholipases on cells only about 20%, but that from antiserum inhibited the effect completely. Similarly, the gamma globulin fraction of normal serum inhibited phospholipase activities on the emulsion only 15 to 33%, although that from antiserum inhibited all four activities (Table 3: PTI, PTE, PTC, SPH) almost completely. The globulin fractions alone had little or no effect on the cells or phospholipids.

The results of testing partially purified phospholipase fractions for their ability to increase the galactosidase activity of 4-hour cells of the cryptic mutant of E. coli are presented in Table 4. Only those fractions that had significant activity on PTE and phosphatidyl choline (PTC) increased the permeability of the EDTA-treated cells markedly, namely the crude material and fraction 24. Combined and concentrated fractions 39 to 41, which had highest activities on phosphatidyl inositol (PTI) and SPH, produced a significant effect on cells only when tested at a higher concentration (14 μg). Fraction 59, which was most active on PTI, was essentially inactive on cells. Table 4 includes data obtained with a commercial preparation of C. perfringens α-toxin known to have phospholipase C activity.[10] The relatively low effect on cell permeability may be related to its small activity on PTE as measured in our test with the emulsion of mixed phospholipids.

TABLE 3. INHIBITION OF PHOSPHOLIPASE ACTIVITY ON E. COLI CELLS AND ON AN EMULSION OF PHOSPHOLIPIDS BY THE GAMMA GLOBULIN FRACTION OF RABBIT ANTISERUM PREPARED WITH THE CRUDE PHOSPHOLIPASES OF B. CEREUS

| Sample[a] | Galactosidase of Treated Cells,[b] absorbancy | Degradation of Phospholipids in a Mixed Emulsion[c] PTI, % | PTE, % | PTC, % | SPH, % |
|---|---|---|---|---|---|
| Phospholipases incubated with NaCl | 0.409 | 89 | 68 | 80 | 23 |
| Phospholipases incubated with normal serum gamma globulin | 0.329 | 60 | 52 | 68 | 19 |
| Phospholipases incubated with antiserum gamma globulin | 0.103 | 9 | 0 | 0 | 0 |
| Normal serum gamma globulin incubated alone | 0.089 | | | | |
| Antiserum gamma globulin incubated alone | 0.103 | 9[d] | 0[d] | 0[d] | 4[d] |

a. Supernatant solutions used after treatment described in Section II.
b. Cells had been harvested at 4 hours. Value for control cells treated for 3 minutes with EDTA and 20 minutes with NaCl was 0.102.
c. Incubated at 37 C for 20 minutes. PTI = phosphatidyl inositol, PTE = phosphatidyl ethanolamine, PTC = phosphatidyl choline, SPH = sphingomyelin.
d. A mixture of the two globulin preparations was used.

TABLE 4. THE RELATIONSHIP BETWEEN THE PHOSPHOLIPASE ACTIVITIES OF CRUDE AND PARTIALLY PURIFIED FRACTIONS OF *B. CEREUS* AND *C. PERFRINGENS* α-TOXIN AND THEIR EFFECTS ON *E. COLI* ML35

| Phospholipases[a] | Degradation of Phospholipids in Mixed Emulsion[b] | | | | | Galactosidase of Treated Cells[c] | |
|---|---|---|---|---|---|---|---|
| | Phospholipase Protein Tested, μg | PTI, % | PTE, % | PTC, % | SPH, % | Phospholipase Protein Tested, μg | Increase, % |
| Crude, *B. cereus* | 10 | 75 | 43 | 70 | 28 | 5 | 57 |
| Fraction 24, *B. cereus* | 8.8 | 0 | 57 | 70 | 7 | 5 | 52 |
| Fraction 39 to 41, *B. cereus*[d] | 8.9 | 38 | 11 | 10 | 54 | 7<br>14 | 8<br>43 |
| Fraction 59, *B. cereus* | 9.2 | 68 | 5 | 14 | 11 | 5 | 3 |
| Commercial *C. perfringens* α-toxin | 50 | 0 | 13 | 55 | 17 | 50<br>100 | 10*<br>26* |
| Crude, *B. cereus* | | | | | | 40 | 219* |

a. Purified as described previously,[1] but the fraction numbers do not correspond to those previously reported.
b. See footnote c of Table 3.
c. Cells were harvested at 4 hours. They were used on the next day for results marked with an asterisk, but were stored for 7 days at 5 C before use in the other tests.
d. These fractions were pooled, concentrated, and dialyzed.

## IV. DISCUSSION

The test system described is very sensitive to ions. Originally, cells were treated and tested for the availability of galactosidase without much dilution. Introduction of ions with various solutions resulted in the stimulation of galactosidase activity and led to erroneously interpreted results. Therefore, the model system was modified so that cells are treated in a relatively concentrated suspension and are diluted 500-fold before galactosidase measurement. Included in the dilution fluid are $1 \times 10^{-4}$ M $MnCl_2$ and 0.05 M 2-mercaptoethanol, which increase the sensitivity of the test system by markedly stimulating the galactosidase above that obtained with either substance alone and which also stabilize the enzyme. A peculiar ionic effect on cell-free galactosidase was noted during development of the model system. Although $1 \times 10^{-4}$ M and higher concentrations of EDTA inhibited the galactosidase activity of a crude extract of E. coli, presumably by chelating an ion that stimulated the enzyme, a twofold to threefold stimulation was obtained with $1 \times 10^{-5}$ M EDTA. No effect was noted with $1 \times 10^{-6}$ M EDTA. The stimulation by $1 \times 10^{-5}$ M EDTA was also evident when $MnCl_2$ was present at final concentrations of $5 \times 10^{-6}$ to $5 \times 10^{-5}$ M, but it disappeared when the $Mn^{++}$ concentration was about 10 times greater than that of the EDTA. The stimulation may have been caused by a preferential chelation of some inhibitory ion present in the dilute, crude extract, so that the galactosidase activity was increased in the absence as well as in the presence of low concentrations of added $Mn^{++}$. It is also possible that the EDTA produced a more direct synergistic effect on activating ions within the described concentration range. Such effects of EDTA and metal ions should be considered when studying changes in permeability to galactosidase substrates, because stimulation of any extracellular as well as of intracellular galactosidase might be misinterpreted as an increase in cell permeability. In the model system described here, the low final concentration of EDTA during the galactosidase test ($2 \times 10^{-6}$ M) and the presence of excess $Mn^{++}$ ($1 \times 10^{-4}$ M) prevent any stimulations due to slight ionic variations introduced with the solutions being assayed. The high dilution also makes unnecessary the removal of cells before reading the yellow product of galactosidase action on ONPG. The fact that denaturing the phospholipases by heat, or inactivating them with antiserum, prevented their effect on the galactosidase activity of the cells showed that the stimulations obtained with the native phospholipases were not merely caused by ions added with these preparations (Tables 1 and 3).

Several methods have been reported for estimating total galactosidase of E. coli. We have found that variable results are obtained that depend on the degree of lysis. If the cells are not completely permeable to any ions present that stimulate galactosidase, low values will be obtained. For this reason we have found that methods depending on lysis by toluene[11]

or toluene plus sodium deoxycholate[12] are not always satisfactory. Variations in estimations may be partly the result of differences in susceptibility to lysis of cells in different stages of the growth cycle or changes in culture conditions.[13] This may also be true for methods depending on cell lysis by EDTA plus lysozyme.[14] With the specific sonic treatment described here, we have obtained reproducible values regardless of cell age. However, the effects of mercaptoethanol on galactosidase activity described by Reithel et al.[9] must be considered for certain strains of E. coli. In any case, it is essential that galactosidase be shown to be stable during any treatment employed for estimating the total enzyme content of cells.

Although the phospholipase fractions that increased the galactosidase activity of the cryptic E. coli cells degraded both PTC and PTE, we suggest that only the latter phospholipid is involved. No PTC has been detected in E. coli, but PTE has been found to be the principal phospholipid.[3] We have verified this for the mutant strain of E. coli ML35 used here. We have made no further attempts to separate the PTE- and PTC-phospholipase C activities of B. cereus.[2] We agree with the description of phospholipase C given by van Deenen,[10] but we feel that several specific enzymes are present in crude preparations from B. cereus[2] that are often implied to have a single phospholipase C with low substrate specificity. Although the phospholipase C of C. perfringens α-toxin was originally thought to be inactive against PTE, it was shown to attack PTE under certain conditions.[15] The commercial preparation that we tested had relatively low activity on PTE compared with PTC in our emulsion of mixed phospholipids. However, the ability to attack PTE may be related to the correspondingly low effect on E. coli permeability (Table 4). In contrast to our results with E. coli cells, we previously found that the ability to release alkaline phosphatase from rabbit bone and kidney cells was associated with the phospholipase that degrades PTI.[2]

EDTA is known to increase the permeability of E. coli to ONPG and to other substances.[16] This is probably associated with the removal of lipopolysaccharide from the cell envelope.[17] We assume that the same mechanism enables the phospholipase of B. cereus to attack the PTE of the cell membrane in our model system. Under our conditions of treatment, EDTA alone not only increases cell permeability but also causes cell lysis (Fig. 1). These effects of EDTA are more marked with relatively young cells (Fig. 3).

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13. ABSTRACT
A permeaseless mutant of Escherichia coli that produces β-galactosidase constitutively was treated briefly with ethylenediaminetetraacetate (EDTA) and then with the phospholipases of Bacillus cereus. An increase in cell permeability and lysis was indicated by an increase in β-galactosidase activity and a decrease in absorbancy of the cell suspension. The susceptibility of the cells to attack by EDTA and the phospholipases was markedly affected by the age of the cells when harvested. The results suggest that the permeability changes are associated with the activity of a phospholipase that specifically degrades phosphatidyl ethanolamine. A sonic-treatment method, which is independent of the age of E. coli cells when harvested, is described for determining their total β-galactosidase content.

14. Key Words

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| Phospholipids | Absorption |
| *Escherichia coli | Susceptibility |
| *Galactose | Sonic treatment |
| Bacillus cereus | *Sequesters |
| Permeability | Lysis |

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